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(21) International Application Number: PCT/FI90/00055 (22) International Filing Date: 27 February 1990 (27.02.90) (30) Priority data: 890949 28 February 1989 (28.02.89) FI (71) Applicant (for all designated States except US): ALKO LTD. [FI/FI]; PB 350, SF-00101 Helsinki (FI). (72) Inventors; and (75) Inventors/Applicants (for US only) : VALO, Risto, Juhani [FI/FI]; Maininkitie 21 A 6, SF-02320 Espoo (FI). HÄGGBLÖM, Max, Mikael [FI/FI]; Topeliuksenkatu 10 B 36, SF-00250 Helsinki (FI). SALKINOJA-SALON- EN, Mirja [FI/FI]; Nastolantie 26, SF-00600 Helsinki (FI).		(74) Agent: OY BORENIUS & CO AB; Arkadiagatan 21 A, SF-00100 Helsingfors (FI). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (Eu- ropean patent), NO, SE (European patent), SU, US. Published <i>With international search report.</i>
(54) Title: PROCESS AND MEANS FOR THE MICROBIOLOGICAL REMEDIATION OF POLLUTED SOIL AND MIC- ROORGANISMS FOR USE IN SAID PROCESS (57) Abstract <p>The invention relates to a process for the microbiological remediation of soil polluted by chlorophenols, the soil to be de- contaminated being composted with the chlorophenol-degrading microorganisms and the conditions being adjusted so that they favour for the growth of the microorganisms. According to the invention such chlorophenol-degrading microorganisms of the ge- nus <i>Rhodococcus</i> and <i>Mycobacterium</i> are preferably used which are capable of degrading the chlorophenols also in field condi- tions. According to the invention the microorganisms are preferably immobilized in a solid porous support.</p>		

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Process and means for the microbiological remediation of polluted soil and microorganisms for use in said process

The invention relates to a process for the remediation of soil polluted by chlorinated phenolic compounds with the aid of microorganisms capable of degrading chlorinated phenolic compounds. The invention also relates to the new microorganisms to be used in said process as well as to said microorganisms immobilized in a solid support.

Chemically polluted soil and water is becoming an increasingly serious environmental problem in the industrialized countries. Chlorinated phenolic compounds and their derivatives, which are referred to in the following specification and claims by the term "chlorophenols" for short, are toxic hazardous contaminants which are hardly degradable in the environment. In Finland chlorophenol contaminated soil causes problems at the sites of saw mills where said chlorophenols have been used and where concentrations up to 5000 to 10 000 mg per kg dry soil have been located. Chlorophenols have also been found in surface water and in ground water in environmentally hazardous contents.

US Patent 4,713,340 (Crawford) describes the purification of chlorophenol contaminated surface and ground water by using bacteria of the genus Flavobacterium deposited under No. ATCC 39723 and capable of degrading chlorophenols. The same Patent also discloses that polluted soil may be purified by leaching the chlorophenols from the soil and removing said chlorophenols from the resulting polluted water.

The purification of polluted water with the aid of chlorophenol-degrading microorganisms has been disclosed in the copending international patent application PCT/FI89/00144 (based on the Finnish patent application 883685 filed August 8, 1988) which is incorporated herein by reference. Said

application discloses a process for the microbiological purification of polluted water by a contaminant-degrading microorganism, in which process chlorophenols are enriched in a chlorophenol accumulating solid support and microorganisms which are immobilized in said support are made to degrade the accumulated chlorophenols. Polyurethane is the preferred support and biofilter which reversibly captures chlorophenols from the water. Said polyurethane may be specifically modified for the immobilization of microorganisms. As the chlorophenol-degrading microorganisms immobilized in the support the water purification process preferably uses chlorophenol-degrading bacteria of the genus Rhodococcus, which are capable of very effectively degrading the chlorophenols in the water.

Other known chlorophenol-degrading microorganisms are for instance some Arthrobacter and Pseudomonas bacteria, which have been disclosed by Portier et al., in Toxicity Assessment: An International Quarterly, Vol 1, p. 501...513, (1986).

The removal of chlorophenols from polluted soil is a much more difficult problem than their removal from water. Several chlorophenol-degrading microorganisms are prior known which are capable of degrading chlorophenols in favourable conditions in water or in a sterilized soil sample in a laboratory. It has, however, been found that it is very difficult to keep these known microorganisms alive in natural conditions in the soil and thus enable them to degrade the chlorophenols in said soil.

Although chlorophenols are toxic to humans, contaminated soil includes plenty of microorganisms which tolerate chlorophenols. Some of these do not affect chlorophenols at all, some transform chlorophenols e.g. by methylating them whereby the resulting chloroanisoles may be even more hazardous to humans than the actual chlorophenols. Only a part of the microorganisms which tolerate chlorophenols are ones which are capable of using chlorophenols as their carbon source.

Even though contaminated soil may include naturally occurring microorganisms which in laboratory conditions are capable of degrading chlorophenols, it has, however, been found that chlorophenols remain almost undegraded in soil. The natural conditions are generally such that they do not favour chlorophenol-degrading microorganisms.

Chlorophenol-degrading microorganisms which occur naturally in contaminated soil have been used for decontaminating polluted soil (Valo et al., Appl. Microbiol. Biotechnol. 1986, 25:68 - 75). In said experiment a naturally occurring microbe population was made to function by raising the temperature of the contaminated soil, adding nutrients to the soil and adjusting the pH close to neutral.

In a parallel experiment in the above mentioned paper it was noted that the degradation of chlorophenols in laboratory conditions accelerated when a pure culture of the chlorophenol-degrading microorganism Rhodococcus chlorophenolicus PCP-1 DSM 43826 was added to a sterilized contaminated soil sample. Said microorganism is described in detail by Apajalahti et al. in Int. J. Syst. Bacteriol., (1986) 36:246 - 251. In the field scale experiment, however, it was found that the addition of chlorophenol-degrading bacteria in an amount of 1×10^5 cells/g of contaminated soil did not have any significant effect.

As mentioned above several chlorophenol-degrading microorganisms are incapable of competing for the vital function's with other more quickly multiplying microorganisms. When such microorganisms are added to a foreign environment they are therefore generally incapable of effectively degrading chlorophenols. Moreover, the temperature of the soil is so low that it does not correspond to the temperature of bioactivity of the microorganisms (generally about 20 to 35 °C).

It has now been found that it is possible to isolate from soil or water certain chlorophenol-degrading microorganisms of the strains Rhodococcus and Mycobacterium which are stable in soil also in naturally occurring conditions. It has, however, been found that the chlorophenol-degrading activity of said microorganisms is so low that without external means they are incapable of decreasing the chlorophenol content of contaminated soil to any significant degree.

In connection with the present invention the conditions for the activity of said chlorophenol-degrading microorganisms were studied and hereby the means were discovered by which the said microorganisms can be made to work effectively so that the contaminated soil can be reclaimed.

The object of the present invention is to provide a process, by which chlorophenol contaminated soil can be efficiently reclaimed with the aid of chlorophenol-degrading microorganisms.

The object of the invention is also to provide a support material for use in said process which support comprises an immobilized microorganism.

The object of the invention is further to provide new microorganisms which are isolated from the environment and which preferably can be used in said process.

The more detailed objects of the invention will appear from the following specification as well as from the appended claims.

Thus, the object of the invention is a process for the microbiological remediation of contaminated soil from chlorinated phenolic compounds and their derivatives with the aid of chlorophenol-degrading microorganisms, in which process chlorophenol-degrading microorganisms belonging to the genus

Rhodococcus and/or Mycobacterium are added to the contaminated soil and the facilities for the chlorophenol-degrading activity of the used microorganisms are improved by adjusting the growth conditions of said microorganisms.

In order to improve the viability of said microorganisms in the conditions of the soil to be decontaminated it has been discovered within the scope of the invention to be advantageous to immobilize them in a solid porous organic support, preferably in polyurethane which is especially modified for this purpose.

According to the invention it has also been found important to improve the facilities for the bioactivity of the microorganisms by adjusting the physical and/or chemical parameters of the soil, such as temperature, pH, moisture, redox potential, nutrients, aeration, etc. In this connection it has surprisingly been discovered that when adding nutrients one should avoid adding a carbon source which is easily utilizable by the microorganisms.

The present invention also relates to a modified polyurethane support which contains immobilized chlorophenol-degrading microorganisms, said support being characterized in that chlorophenol-degrading microorganisms of the genus Rhodococcus and/or Mycobacterium are immobilized into the polyurethane resin during the production of the polymer.

The invention further relates to microorganisms which are isolated from the environment and which degrade chlorinated phenols and their derivatives, and which microorganisms are:

Rhodococcus sp. CG-1, which has been deposited in accordance with the Budapest Treaty on January 10, 1989 in the depository DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and obtained the number DSM 5146, and

Mycobacterium sp. CG-2, which has been deposited in accordance with the Budapest Treaty on January 10, 1989 in the depository DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and obtained the number DSM 5129.

The invention is further illustrated by the following specification and the appended drawings, wherein

Fig 1 shows the effect of the addition of a carbon source on the evolution of carbon dioxide indicating the degradation of pentachlorophenol, PCP, responsive to the chlorophenol-degrading activity of a microorganism immobilized in accordance with the present invention.

Fig 2 shows the degradation of ^{14}C labeled pentachlorophenol, PCP, responsive to the bioactivity of a microorganism according to the invention.

According to the process of the present invention chlorophenols are removed biologically from contaminated soil by adding a sufficient amount, preferably at least 1×10^6 cells/g dry soil of chlorophenol-degrading microorganisms of the genus Rhodococcus and/or Mycobacterium and by at the same time adjusting the conditions of bioactivity for said microorganisms so that an effective microbial activity is possible.

In the invention it has been found especially preferred to add the microorganisms immobilized in a solid porous organic support. The support forms a favourable living environment for the microorganisms and it protects the microorganisms from the attack of predatory beings. It is further preferred that the porous support is capable of accumulating chlorophenols from the liquid phase of the surrounding soil and thus lowering the chlorophenol concentration in the immediate living environment of the microorganisms. This is especially important when it is a question of the microorganisms of the genus Rhodococcus and Mycobacterium used according to the invention since the

tolerance of said microorganisms for chlorophenols is relatively low and their activity is thus improved when the support lowers the chlorophenol content of the water phase of the soil. When the chlorophenol content decreases due to the microbial activity, chlorophenols migrate back from the support into the soil for degradation by the microorganisms.

Porous materials, especially porous organic materials such as wood chips and wood bark and polyurethane are suitable as support materials since they have been found to function well as attachment substrates for the microorganisms and they accumulate chlorophenols.

According to the invention polyurethane resins which are capable of effectively acting as immobilization substrates for the degrading microorganisms are especially well suited as supports. The polyurethanes to be used have a large specific area and a large porosity. The utilized polyurethane resin may be of a special polyurethane kind which is modified for the immobilization of bacteria and which is capable of accumulating chlorophenols due to its large surface area and the active carbon that it contains as well as due to its surface charge.

Chips of wood and wood bark have been found to reversibly accumulate chlorophenols from chlorophenol containing solutions (see Apajalahti et al. Microbiol. Ecol. (1984) 10:359...367) and to function very well also as immobilization substrates for microorganisms.

Such supports comprising immobilized chlorophenol-degrading microorganisms are disclosed in the previously mentioned international patent application PCT/FI89/00144 and they can be used also in the process of the present invention.

In the scope of the present invention it has further been found that the immobilization of the microorganisms into the

polyurethane support may be performed during the production of the support material. The immobilization is then performed during the polymerization of the polyurethane for instance by adding living cells into a mixture of polyethylene glycol and an isocyanate compound. During the polymerization whole cells form a part of the actual polymer. The immobilization of microorganisms by polymerization and polycondensation are known immobilization techniques as such, as is made evident by the book by Klein, J., Wagner, F., Applied Biochemistry and Bioengineering, Vol. 4, in the part called Methods for the Immobilization of Microbial Cells, p. 25 to 31.

When encapsulated within the polyurethane the microorganisms are especially well protected for the purpose of being used according to the present invention. Moreover, the actual polyurethane may be modified to suit the needs of the microorganisms and the chemical to be treated.

The bioactivity of the contaminant-degrading bacteria and especially their degrading rate depends on the temperature. In field conditions the problem with the reclamation of contaminated soil often is that the bacterial activity at a temperature below 15 °C is low and stops completely below 8 °C. In an embodiment of the invention the required heating is provided for instance by allowing for a heat producing composting to take place and by ascertaining that the size and form of the pit are heat energetically favourable and/or by heating the pit by external heating, such as electric resistor elements or the like. The amount of composting material to be added depends on the natural content of organic material in the soil to be treated as well as on the need to raise the temperature. A suitable amount of digestible organic material is generally about 0.1 to 1 %.

The rotting organic material, such as wood bark, park waste etc. functions as an oxygen consuming agent besides providing heat and porosity. Thus the redox potential of the bioactivity

is controlled. In accordance with the invention it has been found that the chlorophenol-degrading activity of the said microorganisms is favoured by a low oxygen content in the soil. Thus for instance 1 % air does not contain a harmful amount of oxygen, while on the other hand there is an excessive amount of air for the microorganisms in case the soil contains more than 50 % air. The soil to be decontaminated is therefore preferably isolated from the environment by a plastic film. This also prevents rain water from penetrating into the compost and chlorophenol containing water from leaking to the lower soil layers. The soil to be decontaminated is preferably mixed at intervals in order to bring the microorganisms into contact with fresh portions of the soil.

For the bioactivity of the microorganisms the pH is adjusted to over 6, preferably close to 7 to 8. The contaminated soil may be buffered for instance by the addition of ash which at the same time comprises a mineral nutrient. In accordance with the invention it has been found that nitrogen and phosphorus should generally be added to the soil.

In the addition of nutrients according to the invention it has surprisingly been found that the addition of an easily metabolized carbon source favours the o-methylation of the chlorophenols and weakens the mineralization of the chlorophenols to CO₂ and inorganic chloride. Thus it is extremely important that no easily metabolized carbon source is added to the soil to be decontaminated.

In order to maintain a sufficient gas circulation in the soil said soil must not be too moist. On the other hand the microorganism activity is not either at its maximum in too dry soil. In the soil reclamation according to the invention it has been found preferable to adjust the moisture of the soil to a value which is close to 60 % of the water holding capacity, WHC, of the soil.

Microorganisms which are useful in the process of the invention are chlorophenol-degrading microorganisms of the genus Rhodococcus and Mycobacterium which comprise for instance the following identified strains:

Rhodococcus chlorophenolicus PCP-1 (DSM 43826), which has been described by Apajalahti et al. in Int. J. Syst. Bacteriol., (1986) 36:246...251, and which has been re-deposited according to the Budapest Treaty on January 10, 1989 in the depository DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and obtained the number DSM 5128;

Rhodococcus sp. CP-2, which is disclosed in the international patent application PCT/FI89/00144 as well as in Häggblom et al. Appl. Envir. Microbiol (1988) 54: 3043...3052, and which has been deposited according to the Budapest Treaty on May 13, 1988 in the depository DSM and obtained the number DSM 4598;

Rhodococcus sp. CG-1, which has been deposited according to the Budapest Treaty on January 10, 1989 in the depository DSM and obtained the number DSM 5146; and

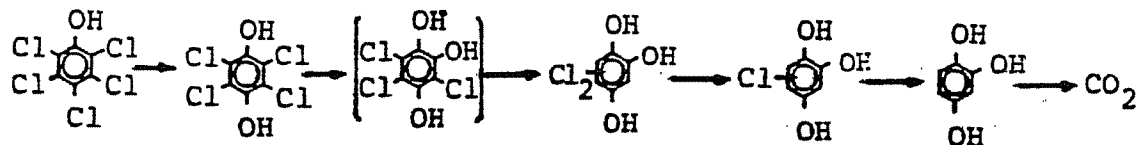
Mycobacterium sp. CG-2, which has been deposited according to the Budapest Treaty on January 10, 1989 in the depository DSM and obtained the number DSM 5129.

Said microorganisms were isolated from chlorophenol contaminated soil and water and it is obvious to a person skilled in the art that other corresponding microorganisms may be isolated in a corresponding way and that such may be used in the process of the invention. It is also clear that chlorophenol-degrading cultures derived from said microorganisms are suitable for being utilized in the process of the invention.

The characteristics of the strains Rhodococcus chlorophenolicus PCP-1 and Rhodococcus sp. CP-2 have been described in the above mentioned literature references, which are included herein by reference.

The strains Rhodococcus CG-1 and Mycobacterium CG-2 which are disclosed as new microorganisms according to the invention have also been described by Häggblom et al. in Appl. Envir. Microbiol (1988) 54: 3043...3052. The reference does not, however, include information as to the deposition of the microorganisms and their identification is therefore not so clear from the said reference that a person skilled in the art could be sure to isolate them and thus obtain them for use. The said microorganisms are therefore to be regarded as being new microorganisms which now by the performed deposition have been disclosed in such a way that they can be regarded as being identified and reproducible. As regards the general characteristics and the chlorophenol-degrading characteristics of the new microorganisms reference is made to the above mentioned paper.

The probable degradation path of pentachlorophenol to carbon dioxide and inorganic chloride by the action of bacteria of the genus Rhodococcus and Mycobacterium is shown in the following reaction scheme:



The chlorophenol-degrading capacity of the bacteria Rhodococcus CP-2 and Rhodococcus chlorophenolicus PCP-1 is genetically stable while the degrading capacity of the bacteria Rhodococcus CG-1 and Mycobacterium CG-2 is instable and vanishes in case the substrate lacks chlorinated phenols. The capacity for degrading chlorinated phenols may be induced into the bacteria either prior to the immobilization or after

the immobilization. In connection with the induction the bacteria are fed with the chemical to be degraded in connection with a nutrient medium so that the bacteria will produce the enzymes which degrade the specific contaminant.

The following Examples illustrate the invention without, however, limiting it in any way.

Example 1

The isolation of chlorophenol-degrading microorganisms

Two 500 ml columns with softwood bark chips as a solid support were inoculated with 10 to 15 g aliquots of a contaminated slurry (sample A) and soil (sample B) each, and 200 ml of a mineral salts medium was percolated through the columns. Tetrachloroguaiacol (TeCG) was weekly added up to a concentration of 10 to 50 μ M. After 3 months, the percolating fluids were found to contain a mixed culture that repeatedly removed added TeCG (10 μ M). The A and B cultures from the percolators were enriched by several dilutions and feeding with 10 to 20 μ M TeCG added at 2 to 3 days intervals. Samples of A and B were streaked onto DSM-65 (glucose 4.0 g, yeast extract 4.0 g, malt extract 4.0 g, H₂O 1000 ml, pH 7.2) agar with 10 μ M TeCG. 130 isolates from each mixed culture were tested for TeCG degradation, by monitoring the removal of TeCG from the liquid. From the culture A was isolated one TeCG degrading colony and it was designated CG-1 and correspondingly a colony isolated from B was designated CG-2.

A third chlorophenol degrading culture (C) was correspondingly obtained from a chlorophenol contaminated soil. The culture in minerals salts medium was enriched for a period of 8 months by repeated dilutions and feeding with PCP (5 mg/l). The culture was plated on yeast extract agar containing PCP (20 mg/l), and one colony that degraded PCP was chosen and designated CP-2.

By corresponding methods also other chlorophenol-degrading Rhodococcus and Mycobacterium bacteria can be isolated and such bacteria may be used in the method and product of the present invention.

The pentachlorophenol-degrading capacity of the isolated microbe strains was measured by using ^{14}C labeled PCP and by monitoring the evolved $^{14}\text{CO}_2$. The degradation of other chlorophenols was also measured.

The strain CG-1 (Rhodococcus sp. CG-1) degraded PCP, 2346-TeCP, 2356-TeCP, 235-TCP, 236-TCP, 246-TCP, 25-DCP, 34-DCP, TeCG, 346-TCG, 346/356 TCG and TCS in 48 hours.

The strain CG-2 (Mycobacterium sp. CG-2) completely degraded PCP, 2345-TeCP, 2346-TeCP, 2356-TeCP, 234-TCP, 235-TCP, 236-TCP, 246-TCP, 24-DCP, 26-DCP, TeCG, 345-TCG, 346-TCG, 346/356-TCG, 456-TCG, 34-DCG, 35-DCG, 36-DCG, TCS and 35-DCS.

CP-2 (Rhodococcus sp. CP-2) also degraded several other chlorinated phenols, guajacols and syringols in 10 μM solutions. In said tests PCP, 2345-TeCP, 2346-TeCP, 2356-TeCP, 234-TCP, 235-TCP, 236-TCP, 245-TCP, 25-DCP, TeCG, 345-TCG, 346-TCG, 356-TCG, 456-TCG, 34-DCG, 35-DCG, 36-DCG, TCS and 35-DCS were degraded in 48 hours.

The above used abbreviations refer to the chlorophenols in the following way: pentachlorophenol (PCP), tetrachlorophenol (TeCP), trichlorophenol (TCP), dichlorophenol (DCP), tetrachloroguaiacol (i.e. tetrachloro-2-methoxyphenol) (TeCG), trichloroguaiacol (TCG), and trichlorosyringol (i.e. trichloro-2,6-dimethoxyphenol) (TCS).

Example 2

Immobilization of the microorganism in the support

Pure cultures of the chlorophenol-degrading microorganisms Rhodococcus chlorophenolicus PCP-1 (DSM 5128) and Rhodococcus CP-2 (DSM 4598), were immobilized in a polyurethane resin mass of the polyurethane REA 90/16 produced by Bayer Ag, West Germany. Said polyurethane is modified for the immobilization of bacteria (polyurethane particle size below 10 mm; density 1.14 to 1.05 kg/l at 20 °C; weight in suspension 115 kg dry matter per m³ of suspension volume; sedimentation rate 94 ± 6 m/h).

A mixed culture of the bacteria was incubated in a fermentor using 1% glucose, 1% soritol as a carbon source and in the presence of a support. As they grew the bacteria excreted slime and attached perceptively onto the surface of the support material. When sufficient biomass had formed the incubation was ended.

The polyurethane mass containing the immobilized Rhodococcus culture was stored for 20 days at +4 °C. In a test performed after said storage the microbes were found to be still capable of degrading chlorophenols.

Chlorophenol-degrading Rhodococcus bacteria have been found to degrade chlorophenols even after having been stored at +4 °C for one year.

Example 3

The degradation of chlorophenols in a soil sample

Two soil samples were used in the test, one of an organic soil and the other of a sandy soil. The unsterilized soil samples were deliberately contaminated with PCP (30 g/l in 0.15 M NaOH) to a concentration of 665 mg/kg dry sandy soil and 1860

mg/kg dry organic soil.

As the chlorophenol-degrading bacterium (degrader) the strain Rhodococcus chlorophenolicus PCP-1 (DSM 5128) was used and as the o-methylating bacterium (methylater) the strain Rhodococcus rhodochrous (DSM 43241) was used. The strains were grown in DSM-65 medium for a week in the presence of sterile polyurethane, PU, (REA 90/16, Bayer, Leverkusen, West Germany). The Rhodococcus chlorophenolicus cultures were induced with PCP daily up to a concentration of 50 μ M.

In the inoculation of the microorganisms 0.5 g (dry weight) of the resulting polyurethane containing the immobilized cells was used. Added to the soils this gave a concentration of 1×10^8 cells/g dry weight for the sandy soil and 2.5×10^8 cells/g dry weight for the organic soil. Sterile PU was used as a control to indicate whether the addition of organic matter as such affects the degradation of PCP.

Glucose/yeast extract was used as an easily metabolized carbon source in the form of a solution of 25 % by weight of glucose and 25 % by weight of yeast extract in water. The test included a total of the following 10 samples:

	<u>Glucose/yeast extract</u>
1) No inoculation	-
2) No inoculation	+
3) Sterile PU	-
4) Sterile PU	+
5) Inoc. with methylaters	-
6) Inoc. with methylaters	+
7) Inoc. with degraders	-
8) Inoc. with degraders	+
9) Inoc. with meth. and degr.	-
10) Inoc. with meth. and degr.	+

All inoculations were made using 1 % by weight of dry polyurethane calculated on the basis of moist soil. Glucose/yeast extract was added to a concentration 0.5 % each relative to the moist soil.

All tests were duplicated with added ^{14}C -labeled PCP. From said tests the degradation of PCP was monitored on the basis of the percentage of evolved $^{14}\text{CO}_2$. The results are shown in Fig. 1, wherein the tests performed with sterile PU and the methylators have been left out for clarity, as the results of said tests essentially followed the same pattern as the corresponding uninoculated soils. In the Fig. the graphs are numbered according to the numbering of the above Table whereby the (+) and (-) marks designate the presence and the absence of a carbon source, respectively. The results clearly indicate that the used microorganism is capable of degrading PCP in unsterilized soil and that the addition of an easily metabolized carbon source decreases the mineralization of PCP.

Example 4

A test corresponding to Example 3 was performed on soil samples (samples A and B) taken from two different sites of chlorophenol contaminated soil. A cell suspension of the degrading microorganism Rhodococcus CP-2 was added to said samples in a concentration of 1.1×10^7 cells/g dry soil. In order to indicate degradation, ^{14}C -labeled PCP was added to the soil samples and the degradation thereof was monitored by measuring the amount of $^{14}\text{CO}_2$ produced. To some of the samples glucose/yeast extract was added in a concentration of 1 % as an easily metabolized carbon source.

The results are shown in Fig. 2 which clearly indicates the negative effect of an easily metabolized carbon source on the mineralization of PCP.

Example 5

Field experiment

Chlorophenol contaminated soil is moved to a plastic lined windrow and the following materials are mixed therein

- wood bark and park refuse in order to raise the content of digestible organic matter to about 0.5 %;
- ash for buffering the pH to a value of about 7;
- fertilizer in an amount of about 1 kg/m³ dissolved in water, said fertilizer containing about 16 % N, about 7 % P and about 3.3 % K;
- water at need in order to adjust the moisture level to about 60 % of the water holding capacity.

Microorganisms Rhodococcus chlorophenolicus PCP-1 and/or Rhodococcus CG-1 immobilized in polyurethane are also mixed into said soil in a concentration of about 10⁸ cells/g dry soil.

After mixing the soil is covered with a plastic film. The temperature of the windrow is monitored during the composting and in case the heat obtained from the digestion is not sufficient to raise the temperature of the windrow to about 20 to 25 °C an additional heating device mounted in the bottom of the windrow is used for heating. The soil is mixed and its chlorophenol content is measured about once a month. When the chlorophenol content of the soil has decreased to a pre-determined acceptable level the windrow is broken up and the reclaimed soil is moved away.

Claims

1. A process for the microbiological remediation of polluted soil from chlorinated phenolic compounds and their derivatives with the aid of chlorophenol-degrading microorganisms, characterized in that chlorophenol-degrading microorganisms of the genus Rhodococcus and/or Mycobacterium are added to the contaminated soil in cell counts which are sufficient to provide a chlorophenol-degrading bioactivity and that the facilities for the bioactivity of the used microorganisms are improved by adjusting the growth conditions for said microorganisms.
2. A process according to claim 1, characterized in that the microorganisms are added immobilized in a solid porous support.
3. A process according to claim 2, characterized in that the microorganisms are immobilized in a polyurethane resin, in wood bark or in wood chips.
4. A process according to claim 3, characterized in that the microorganism are immobilized in a polyurethane resin by incubating said microorganisms in a polyurethane containing medium or by producing said polyurethane from a microorganism containing medium.
5. A process according to one of the preceding claims 1 to 4, characterized in that the facilities for the bioactivity of said microorganisms are improved by adjusting the physical and/or chemical parameters of the soil, said parameters being chosen from the group consisting of temperature, pH, moisture, redox potential, nutrients, aeration, etc.

6. A process according to claim 5, characterized in that when adjusting the nutrients the addition of an easily metabolized carbon source is avoided.

7. A process according to claim 5, characterized in that the temperature and the redox potential are adjusted by adding to the soil organic matter, such as microbiologically digestible wood bark, park refuse or the like and/or that the soil to be treated is enclosed in a impermeable plastic film to decrease the aeration.

8. A process according to any one of the preceding claims, characterized in that the microorganisms are chosen from the group consisting of the chlorophenol-degrading microorganisms Rhodococcus chlorophenolicus PCP-1 (DSM 5128), Rhodococcus sp. CP-2 (DSM 4598), Rhodococcus sp. CG-1 (DSM 5146) and Mycobacterium sp. CG-2 (DSM 5129).

9. A polyurethane support modified for the immobilization of bacteria which support contains immobilized chlorophenol-degrading microorganisms, characterized in that chlorophenol-degrading microorganisms of the genus Rhodococcus and/or Mycobacterium are immobilized in said polyurethane resin during the production of the polymer.

10. A polyurethane support according to claim 9, characterized in that the microorganisms are chosen from the group consisting of the chlorophenol-degrading microorganisms Rhodococcus chlorophenolicus PCP-1 (DSM 5128), Rhodococcus sp. CP-2 (DSM 4598), Rhodococcus sp. CG-1 (DSM 5146) and Mycobacterium sp. CG-2 (DSM 5129).

11. A pure culture of a microorganism which is capable of degrading chlorinated phenolic compounds and their derivatives, characterized in that said microorganism is Rhodococcus sp. CG-1 (DSM 5146) or a culture

derived therefrom.

12. A pure culture of a microorganism which is capable of degrading chlorinated phenolic compounds and their derivatives, characterized in that said microorganism is Mycobacterium sp. CG-2 (DSM 5129) or a culture derived therefrom.

1/2

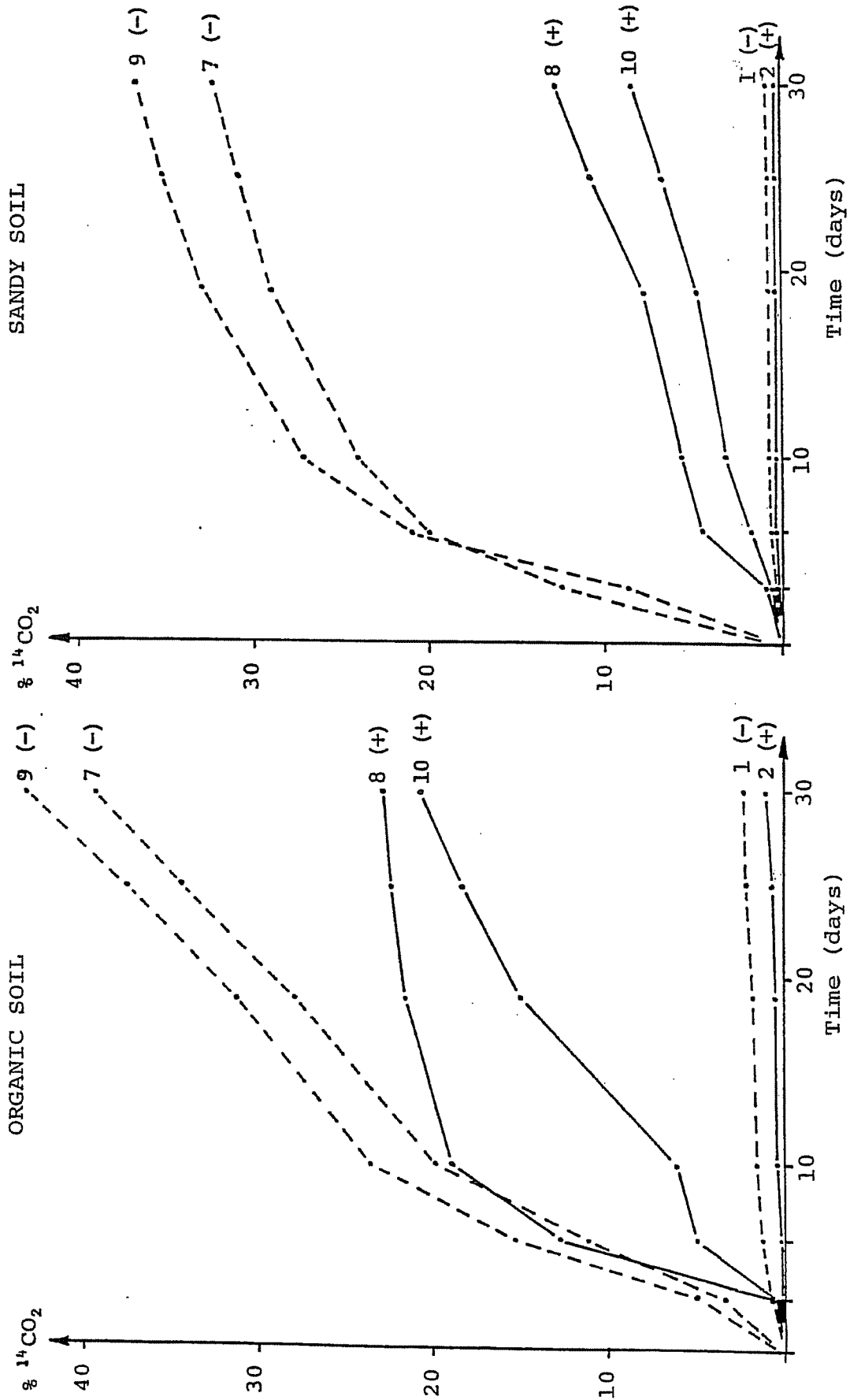


FIG. 1

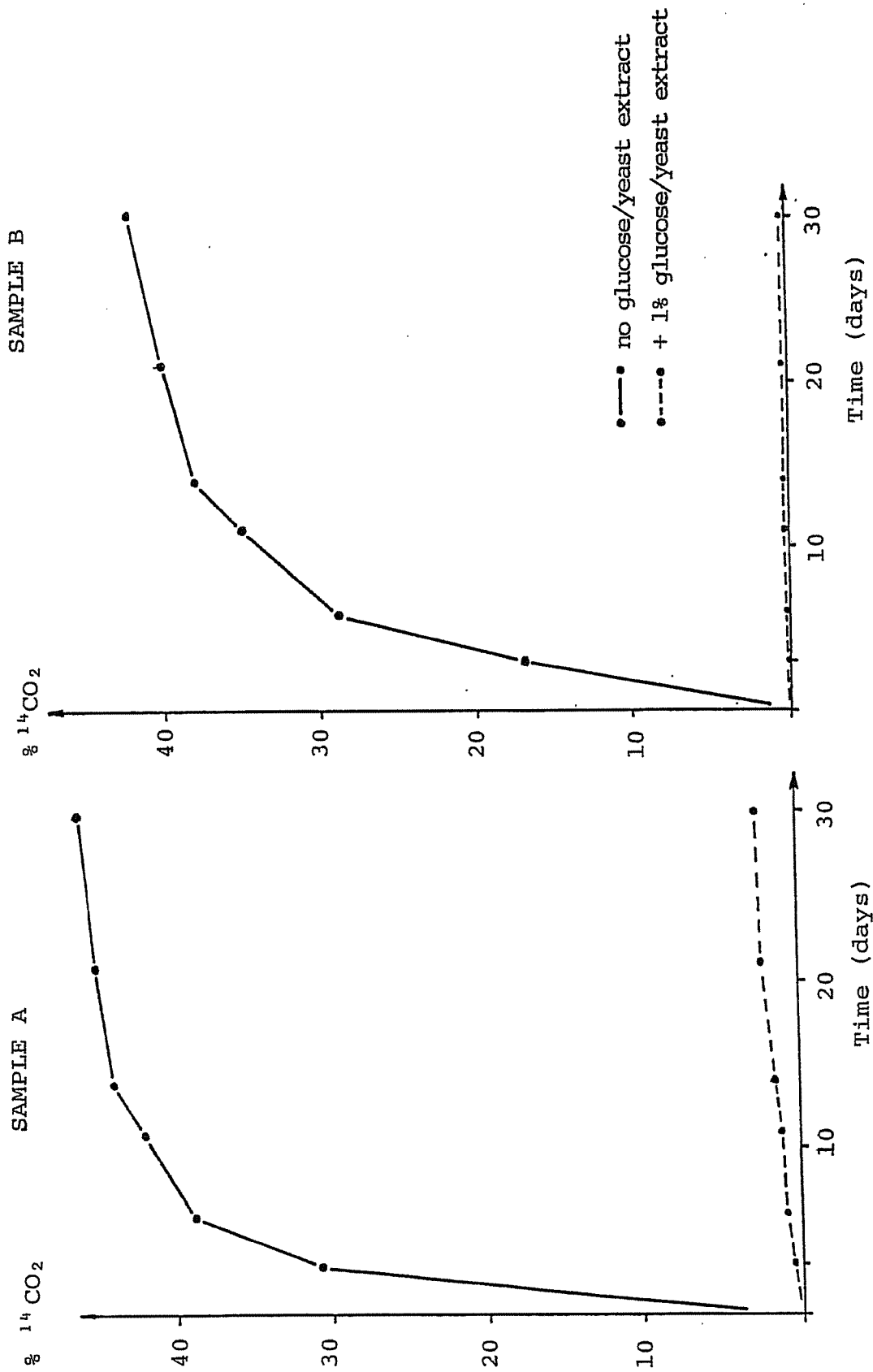
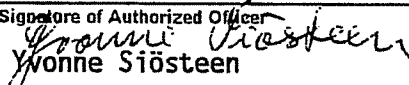


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No **PCT/FI 90/00055**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 P 1/04, C 02 F 3/34, C 12 N 1/20		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	C 02 F; C 12 N; C 12 P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	WO, A1, 90/01465 (ALKO LTD) 22 February 1990, see example 2 --	1-12
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Vol. 54, No. 12, 1988 Max M. Häggblom et al: "Degradation and O-Methylation of Chlorinated Phenolic Compounds by Rhodococcus and Mycobacterium Strains ", see page 3043 - page 3052	1-2,5- 12
Y	--	3-4
Y	DE, A1, 3617875 (HITACHI PLANT ENGINEERING & CONSTRUCTION CO., LTD) 8 January 1987, see page 2, line 66 --	3-4,9- 10
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th May 1990	1990 -06- 07	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	 Yvonne Siösteen	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	APPL MICROBIOL BIOTECHNOL, Vol. 25, 1986 R. Valo et al: "Bioreclamation of chlorophenol-contaminated soil by composting",	1,5-7
Y	--	2-4,8-12
X	Chemical Abstracts, volume 105, no. 5, 4 August 1986, (Columbus, Ohio, US), Apalajahti, Juha H. A. et al : "Rhodococcus chlorophenolicus sp. nov., a chlorophenol-mineralizing actinomycete", see page 411, abstract 39171t, & Int.J.Syst.Bacteriol. 1986, 36(2), 246- 251K	1
A	Chemical Abstracts, volume 108, no. 5, 1 February 1988, (Columbus, Ohio, US), Apajalahti Juha H.A. et al : "Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced Rhodococcus chlorophenolicus ", see page 332, abstract 34580c, & J.Bacteriol. 1987, 169(11), 5125-5130K	1
A	Chemical Abstracts, volume 108, no. 25, 20 June 1988, (Columbus, Ohio, US), Apajalahti, Juha H.A. et al : "Hydroxylation and dechlorination of chlorinated guaiacols and syringols by Rhodococcus chlorophenolicus ", see page 302, abstract 218784d, & Appl.Environ.Microbiol. 1988, 54(3), 683- 687K	1
A	Chemical Abstracts, volume 109, no. 16, 17 October 1988, (Columbus, Ohio, US), Haggblom Max M et al : "Degradation of chlorinated phenolic compounds occurring in pulp mill effluents ", see page 337, abstract 134439c, & Water Sci. Technol. 1988, 20(2), 205- 208K	1-12
A	Chemical Abstracts, volume 106, no. 19, 11 May 1987, (Columbus, Ohio, US), Apajalahti, Juha H. A. et al : "Dechlorination and para-hydroxylation of polychlorinated phenols by Rhodococcus chlorophenolicus ", see page 368, abstract 152724j, & J. Bacteriol. 1987, 169(2), 675- 681K	1-12

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	<p>Chemical Abstracts, volume 106, no. 19, 11 May 1987, (Columbus, Ohio, US), Valo, R et al : "Bioreclamation of chlorophenol-contaminated soil by composting ", see page 269, abstract 151604h, & Appl. Microbiol. Biotechnol. 1986, 25(1), 68- 75K</p> <p style="text-align: center;">-- -----</p>	1-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/FI 90/00055

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 90-05-07
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9001465	90-02-22	NONE	
DE-A1- 3617875	87-01-08	JP-A- 62003787	87-01-09
		US-A- 4791061	88-12-13
		JP-A- 62061583	87-03-18